

Continuous Antibiotic Fermentation—Design of a 20 litre, Single-stage Pilot Plant and Trials with Two Contrasting Processes*

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Summary. The feasibility of producing antibiotics by single-stage continuous fermentation was explored by means of a specially designed pilot plant. Both the chloramphenicol and penicillin processes appeared adaptable to such an operation. At dilution rates of 1.0 and 0.5 volume changes per day respectively, yields of from $\frac{1}{4}$ to $\frac{1}{2}$ of the maxima obtained in batch operation were maintained in the steady-state for more than 2 weeks.

Introduction

Although the potential advantages of continuous fermentation are widely recognized and the theoretical basis is reasonably established, industrial use of the operation has been restricted almost entirely to yeast and yeast products, vinegar, and sewage treatment processes, all of which yield bulk products and depend for their advantageous operation on a highly restrictive growth situation.^{1,2} Future applications of continuous culture, as judged by current trends in research and development,^{3, 4} will probably extend into the production of fine chemicals and into fermentations where culture purity is much more precarious.

One such application of considerable potential importance is in antibiotic production. Since the open literature carried many but only brief notes of progress in this area (see later), we undertook a study to determine the feasibility of continuous antibiotic fermentation. This report describes the design of a 20 l.,

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single-stage pilot plant and the outcome of fermentation trials of two contrasting antibiotic processes, chloramphenicol and penicillin.

Pilot Plant Design and Operation

We sought to design a fermentation unit which would be as simple as possible and potentially capable of scale-up to existing production equipment. The size, 20 l. working capacity, was consistent with usual practice and with the requirements of sampling and adequate feed pumping. The materials of construction were chosen with a view not only to economy but to their specific suitability. For instance, black iron pipe was used for air and steam lines, but the fermentor vent line was constructed of stainless steel because the surging of liquid within this line might otherwise build up high concentrations of iron in the culture. The fermentor and sterilizer vessels were made of Inconel-clad stainless steel. Feed lines were gum-rubber tubing.

A wide-angle view of the installation at one stage of its design is shown in Fig. 1, and a schematic diagram in Fig. 2. The feed

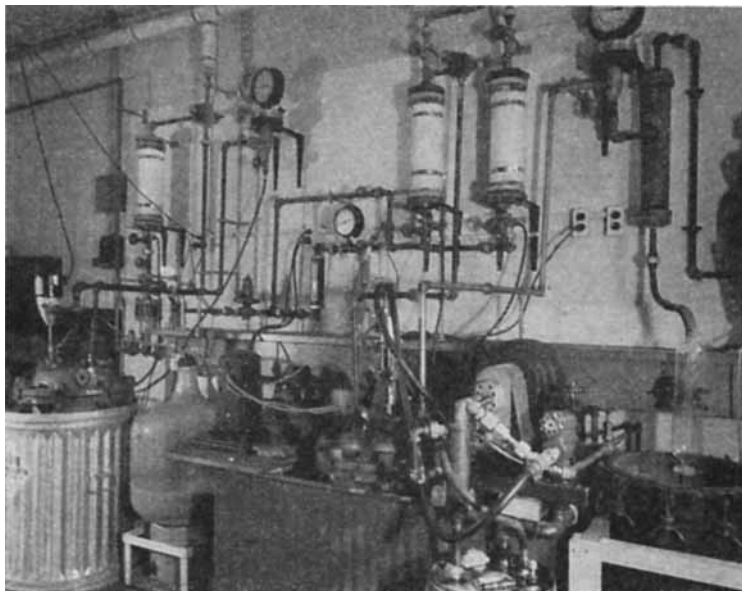


Fig. 1. Wide-angle view of pilot plant installation

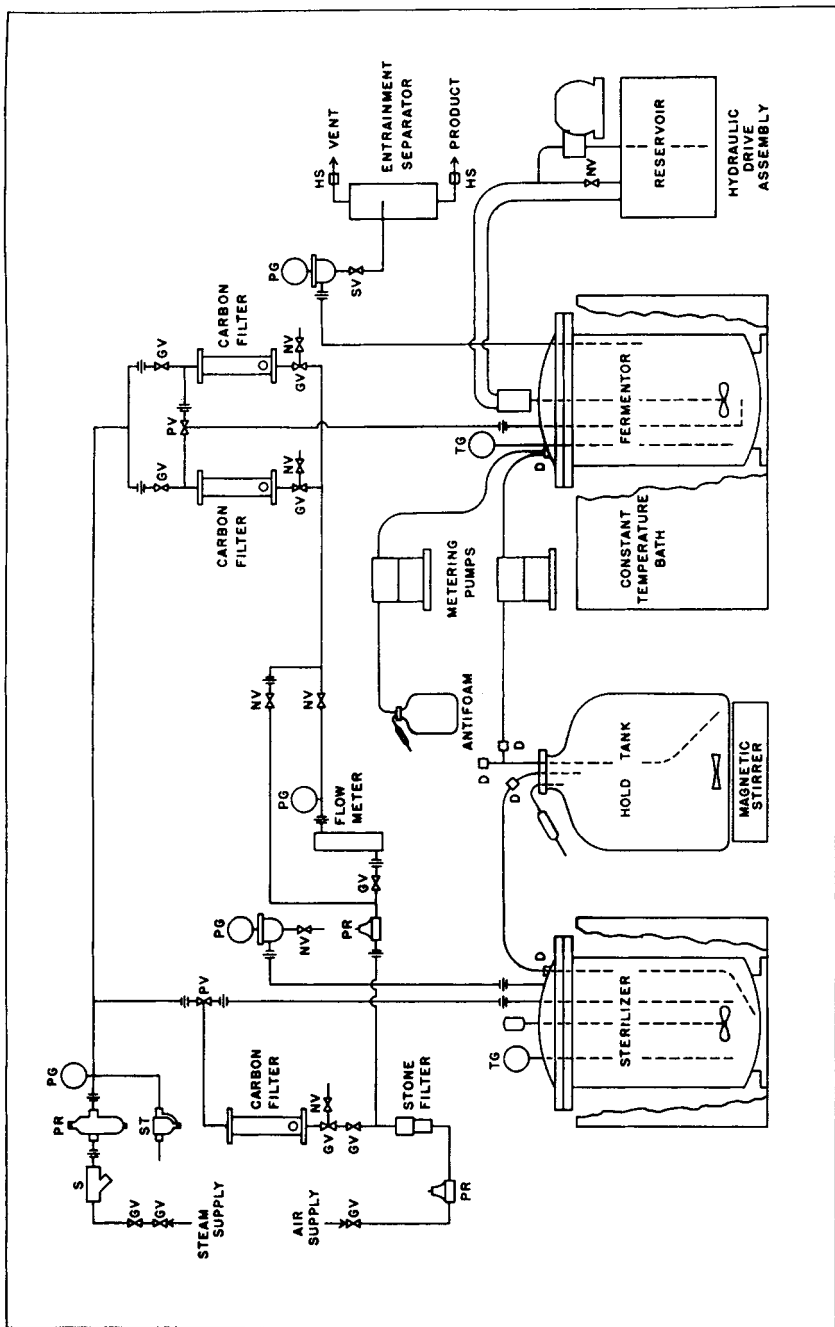


Fig. 2. Schematic diagram of pilot plant

GV, gate valve; NV, needle valve; SV, Saunders-type valve; PV, plug valve; PR, pressure regulator; PG, pressure gauge; TG, temperature gauge; ST, steam trap; S, strainer; HS, heat seal; D, diaphragm

system was operated by first preparing a batch of medium and cooking it under pressure in the sterilizer vessel. The medium then was transferred by means of differential air pressure to the separately sterilized hold tank, a magnetically stirred, 13 gal Pyrex carboy. From this reservoir, the sterile medium was continuously metered into the fermentor by a peristaltic-action Sigma pump. Large hypodermic needles in the connecting feed lines and self-sealing rubber diaphragms fitted to the vessels permitted isolating the hold tank and fermentor from the sterilizer and also replacing the feed lines if necessary during operation.

The air supply to the fermentor was successively stone-filtered, metered, and sterilized by passage through an activated-carbon filter. Duplicate insulated filters (Fig. 3) were piped so as to

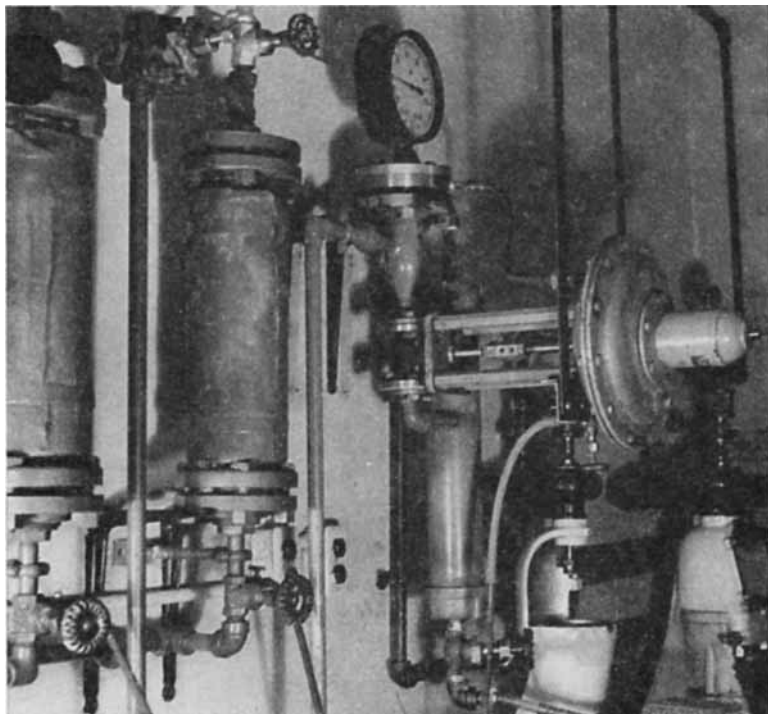


Fig. 3. Close-up view of air filters (left), back-pressure regulating unit (centre), and entrainment separator (behind and below regulator)

allow alternate operation and steam sterilization on a 3-day cycle. The problem of occasional combustion of the carbon packing was solved by adding stainless steel wool to dissipate localized heat and by inspecting the packing through plugs in the bottom of the filters. The sterile air was passed into the contents of the fermentor through a $\frac{3}{8}$ in. stainless steel pipe opening $\frac{1}{2}$ in. below the centre of the impeller.

The fermentor vessel was fitted with a diaphragm adapter at the top for inserting the feed lines, and also near the bottom for sampling the contents by means of a sterile hypodermic syringe and needle. The contents of the fermentor were kept at constant temperature by means of an electrical thermoregulator and saber-heating unit acting against a constant flow of cold water in the water bath. A hydraulically driven turbine positioned on top of the fermentor drove the agitator shaft and conventional four-bladed impeller. The agitator shaft entered the vessel through a stuffing box packed alternately with $\frac{1}{4}$ in. teflon-impregnated asbestos rings and $\frac{1}{2}$ in. teflon spacers; mechanical seals were also attempted but proved less satisfactory. With the possibility of agitator speeds up to 700 rev/min and with the fermentor equipped with four vertical baffles, fully adequate agitation could be attained.

Both air and product were withdrawn through a pipe that extended into the fermentor to the liquid level. Maintenance of a constant liquid level in the fermentor with such an air-lift system required close control of foaming and this was accomplished with the continuous addition of antifoam agent. A separate sterile reservoir and continuous feed line for antifoam agent were necessary in the penicillin process, but a stable silicone emulsion could be incorporated into the medium in the chloramphenicol process. A spring-loaded, back-pressure regulating valve and a product-air separator were located in the effluent system and are shown in Fig. 3. The pressure regulator maintained a positive pressure of about 5 lb/in² gauge in the fermentor independent of the rate of flow of medium or air. Electric heating tapes used as heat seals were located on the effluent air and product pipes to prevent possible back-growth of contaminating micro-organisms along the pipe walls.

Chloramphenicol Fermentation

Chloramphenicol appears to be ideally adapted to continuous fermentation in a single-stage unit, because the batch fermentation exhibits parallel growth and antibiotic formation and, in addition, is favourably characterized by only moderate pH changes, a fluid cell suspension, wide-spectrum effectiveness against contamination, and stable product. As potential disadvantages, the hyphae of the chloramphenicol-producing *Streptomyces venezuelae* tend to fragment and lyse during the latter stages of the batch growth cycle, and the organism is understood to have a moderately high frequency of reversion to mutants with low synthetic ability. Details of the batch chloramphenicol process have been published.^{23, 24} To our knowledge, continuous fermentation of this antibiotic has not been attempted, although there have been brief reports or patents on continuous fermentation of streptomycin,⁴⁻⁶ subtilin,¹¹ and penicillin (see below); these are discussed more fully elsewhere.¹²

Assay procedures. Product samples were withdrawn directly from the fermentor and frozen until a number of assays for antibiotic content could be made together. Chloramphenicol was determined by the method of Glazko *et al.*¹³ The aromatic nitro group of chloramphenicol was quantitatively reduced to a primary amine, which was then determined colorimetrically by the standard Bratton-Marshall diazotization procedure. Correction was made for non-specific aryl amines, and a standard curve was included with each set of determinations. The method measures both the active antibiotic and inactive degradation products; the latter, however, are believed to be present only in a very low concentration, and in any event should remain relatively constant in amount during continuous fermentation.

The pH and percentage of total solids in the crude product were estimated at the time of sampling. The solids content was determined on a volume basis after centrifuging a 10 ml sample for 10 min at 2000 × g.

Inoculum preparation. A culture of *Str. venezuelae* strain 05072 was obtained from Parke, Davis and Company; this strain can produce about 400 µg of chloramphenicol per ml. Soil stocks of the organism were prepared from mature spores after growth

on Anderson's sporulation agar.⁷ When a fermentation was begun, the spore stock was inoculated into 100 ml of chloramphenicol medium II,⁷ modified as shown in Table I, in a 250 ml Erlenmeyer flask. This was incubated on a rotary shaker at 28°C for 48 h and then used as inoculum for a larger 600 ml culture in a 2 l. flask. Three such flask cultures (7.5 volume-per cent) were used to inoculate the fermentor.

Batch chloramphenicol fermentation. A series of batch fermentations were conducted to establish a reference picture in our equipment and with our operating procedures. The fermentor contained 20 l. of the modified chloramphenicol medium (Table I)

Table I. Composition of fermentation mediums

Chloramphenicol medium*		Penicillin inoculum medium†		Penicillin fermentation medium†	
Ingredients	g/l.	Ingredients	g/l.	Ingredients	g/l.
Glycerol (ml/l.)	10	Glucose	20	Lactose	55
Brewers yeast	15	Corn-steep solids (dry weight)	10	Glucose	5
Butanol-acetone fermentation residue	5	CaCO ₃ (total)	5	Corn-steep solids (dry weight)	45
NaCl	5	ZnSO ₄	0.1	CaCO ₃	5
CaCO ₃	1	K ₂ HPO ₄	0.5	ZnSO ₄	0.05
NaOH to pH 7.3-7.4		NaNO ₃	1	KH ₂ PO ₄	0.5
q.s. tap water		MgSO ₄	0.1	Na ₂ SO ₄	0.5
		q.s. tap water		Potassium phenyl acetate	0.5
				Crude lard oil	3
				q.s. tap water	

* Provided by Parke, Davis and Company.

† Provided by Eli Lilly and Company.

with 1 ml of General Electric No. 60 silicone-emulsion defoamer; air was supplied at a rate of $\frac{3}{4}$ volumes per volume of medium per min; the agitator was driven at a speed of 250 rev/min, and the temperature was regulated at 28°C.

Results of a representative batch chloramphenicol fermentation under these conditions are shown in Fig. 4. The maximum yield of 400 µg/ml in 4 days is relatively good for this strain. This particular experiment also was selected to demonstrate that even the early appearance of contamination sometimes has no apparent effect on chloramphenicol formation, a useful

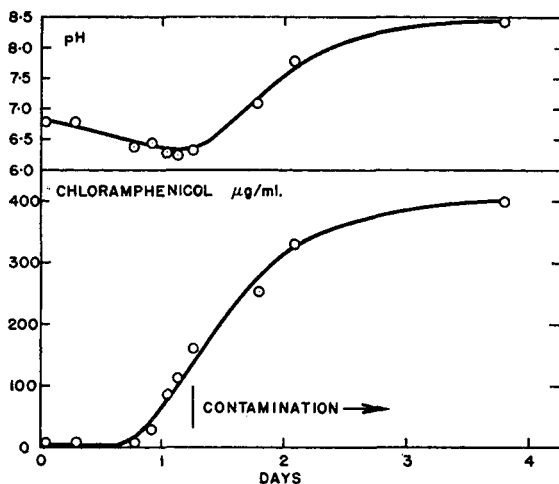


Fig. 4. Batch chloramphenicol fermentation

characteristic for continuous fermentation. However, the abnormal pH changes may have been caused by the contamination.

Continuous chloramphenicol fermentation. A continuous fermentation was begun in the same manner as a batch fermentation, allowed to progress for 24 h, and then put on continuous feed with the same medium. Results of such a trial are shown in Fig. 5.

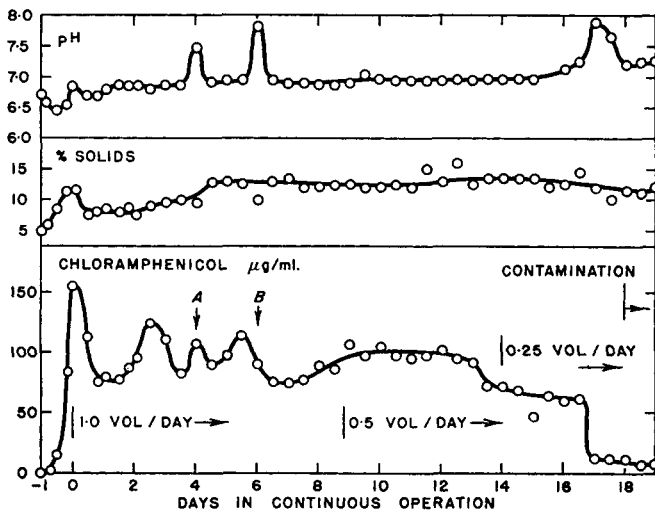


Fig. 5. Continuous chloramphenicol fermentation

It was found that a dilution rate of 1.0 volume changes per day brought an initial drop from the higher batch level and then allowed maintenance of about one-quarter of the maximum batch yield. In three confirmatory experiments of shorter duration with the same dilution rate, yield levels again dropped initially and then were maintained at between 80 and 110 $\mu\text{g}/\text{ml}$. As shown in Fig. 5, lower feed rates tended, if anything, to reduce the yield, although the pH and solids were relatively unaffected. The yield fluctuations observed in this and other continuous fermentations usually could be traced to some operational deviation and did not appear to be inherent in the method. For example, at points *A* and *B* on Fig. 5, the feed pump was inadvertently stopped for 5 and 10 h, respectively. The appearance of contamination after 18½ days of continuous operation apparently had caused the yield to drop, and the experiment was terminated after 20 days.

The question was raised whether the drop in chloramphenicol production might be due rather to 'strain degeneration', especially since a lower yield level unpredictably accompanied the lowering of the feed rate in the third week. Accordingly, the organism was isolated from samples taken at the start of continuous operation as well as after 17 days in this experiment, and after 8 days in another experiment. Each of these cultures was then used to inoculate duplicate shaker flasks of medium and the resulting batch fermentations were analysed daily over a 10-day period. These results are given in Table II. Although the fermentations

Table II. Batch fermentation tests* of inoculums isolated initially and terminally from continuous chloramphenicol fermentations

Hours of incubation	Inoculum from Exp. A						Inoculum from Exp. B (Fig. 5)					
	0 days			8 days			0 days			17 days		
	pH	% Solids	μg Chlor.	pH	% Solids	μg Chlor.	pH	% Solids	μg Chlor.	pH	% Solids	μg Chlor.
48	7.05	5	3	6.95	5	2	7.00	4	0	7.00	5	3
72	7.20	7	18	7.18	7	25	7.15	5	0	7.20	5	4
96	7.55	8	36	7.40	9	37	7.40	7	16	7.35	7	17
120	7.75	10	47	7.65	9	45	7.65	7	24	7.50	8	35
144	7.85	11	50	7.90	10	55	7.90	8	40	7.85	8	44
168	8.10	10	58	7.95	12	63	8.00	8	44	7.95	8	50
192	8.15	11	68	8.00	15	72	8.15	8	48	8.05	9	51
216	8.30	13	68	8.20	15	76	8.25	8	47	8.30	10	50
240	8.45	14	67	8.40	15	72	8.40	8	37	8.40	10	45

* Values given are averages of duplicate determinations.

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were in all cases more protracted and gave lower yields than usual, differences attributable to 'strain degeneration' from continuous culture were not apparent.

Penicillin Fermentation

In contrast to chloramphenicol, penicillin characteristically is formed after mycelial growth has approached its maximum, suggesting that the usual batch cycle is composed of two phases.³² Consequently, penicillin production under steady-state conditions should occur to best advantage in at least a two-stage system in which the growth and production phases could be separated and possibly enhanced individually. At best, single-stage continuous penicillin fermentation should represent only a low resultant of the two phases balanced at the steady-state. Although this outlook has generally prevailed, a recent note by Somerson and Nunheimer³⁰ indicated that factors in the medium, rather than the mould mycelium, are responsible for the limitation of penicillin biosynthesis early and late in the batch cycle; thus, continuous penicillin fermentation in a single-stage system might be expected to be successful.

In further contrast to the chloramphenicol batch process, the penicillin fermentation is characterized by greater pH changes, a thick mycelial suspension, antibiotic activity primarily against Gram-positive bacteria, and a product that is unstable to both temperature and acid as well as to the penicillinase often produced by contaminating bacteria. The *Penicillium* mycelium tends neither to lyse nor to form spores in deep liquid culture.

A considerable amount of literature exists on the penicillin batch process,³² but continuous fermentations have been reported only in brief notes or as patents. Abraham¹ and subsequently others^{14, 19, 20, 31} have devised systems for continuously passing fresh medium beneath surface cultures or¹⁷ inside a Cellophane or porcelain tube on which the organism was grown. Attempts at single-stage continuous submerged fermentation have been made in shaving-packed generators^{2, 28} and in more conventional 30 l. fermentors,^{16, 27} in which yields of 500 to 800 units per ml were maintained for 5 days. Several semi-continuous operations have been described in which a portion of a completed

batch is retained in the fermentor as starter for a new cycle.^{10, 26, 33} A patent has been awarded for a two-stage continuous penicillin process in which overflow from a primary propagator is fed to a secondary fermentor.¹⁸

Assay procedures. The procedures for sampling and for determining pH and percentage of solids in the penicillin fermentations were the same as in the chloramphenicol process. Total carbohydrates were determined by the standard anthrone reaction. Penicillin was assayed by a paper-disc, zone-inhibition test similar to that recommended by the U.S. Food and Drug Administration.⁹ Commercial dehydrated media (Difco) with strain H of *Staph. aureus* (obtained from Eli Lilly and Company) as the test organism were used in the assay procedure.

Inoculum preparation. A lyophilized spore suspension of *Penicillium chrysogenum* strain N-329 was obtained from Eli Lilly and Company; this strain can produce about 2,000 units per ml but has a comparatively protracted fermentation period. Slant spore stocks of the mould were prepared by inoculating the lyophilized spores on Moyer's sporulation medium,²¹ incubating the cultures 8 days at 27°C, and storing them in the refrigerator until needed. To begin a fermentation, spore suspension from one such tube was inoculated into 2 l. Erlenmeyer flasks containing 500 ml of Czapek-Dox corn-steep liquor medium²⁵ modified for inoculum growth as shown in Table I. After incubation at 25 to 28°C on a shaker for 40 to 48 h, three such cultures (7.5 volume-per cent) were used to inoculate the fermentor.

Batch penicillin fermentation. As with the chloramphenicol experiments, a series of batch penicillin fermentations were conducted initially. The fermentation medium used (Table I) was typical of the production media reported in the literature, and included dried corn-steep liquor solids, potassium phenyl acetate, and lard oil. Air was supplied at a rate of 1.0 volume per volume of medium per min, the agitator was operated at a speed of 550 rev/min, and the temperature was regulated at 26°C.

Results of a representative batch penicillin fermentation under these conditions are shown in Fig. 6. The maximum yield of 1,800 units per ml in 7 days is representative for this strain, but the usual appearance of mycelial growth before the penicillin formation was not observed and the pH curve is atypical.

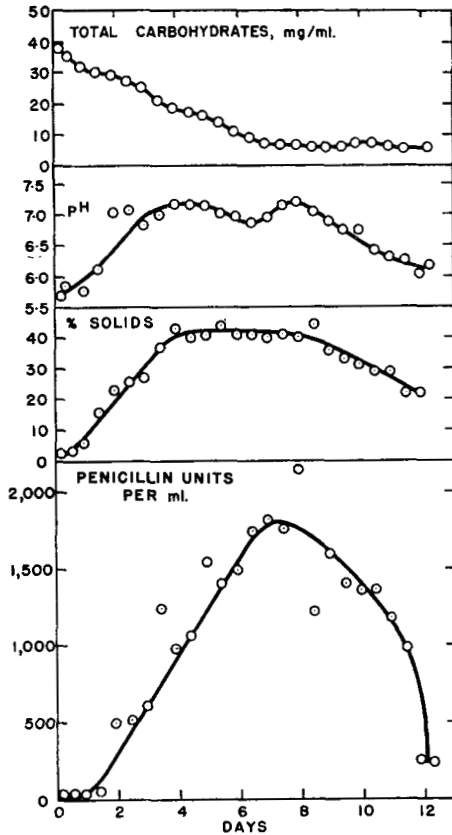


Fig. 6. Batch penicillin fermentation

Continuous penicillin fermentation. A continuous fermentation was begun in the same manner as a batch fermentation, allowed to progress for 48 h, and then put on continuous feed with the same medium but with the lard oil fed separately. The first continuous run was made with a dilution rate of 1.0 volume changes per day, and as shown by the results in Fig. 7, both the mycelium and the penicillin level initially rose but subsequently were washed out after $4\frac{1}{2}$ days of continuous operation. At point *A* on Fig. 7, the feed was stopped for 6 h and then was resumed at point *B*; at point *C* the feed again was stopped for the duration

of the run. The rises in antibiotic level that occurred at the points when the fermentation was allowed to proceed batch-wise indicated that the mould had not lost its ability to synthesize penicillin during the $4\frac{1}{2}$ days of continuous culture. Two additional problems presented themselves in this first experiment: a heavy growth of eventually sporulating mycelium

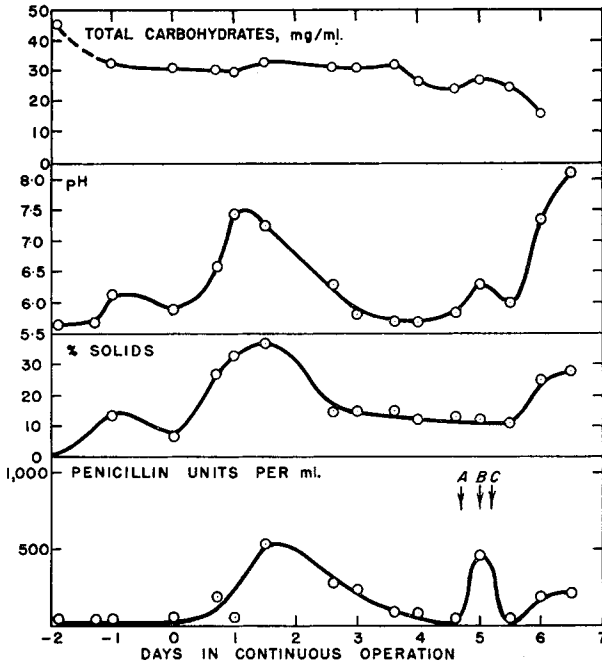


Fig. 7. Continuous penicillin fermentation at 1.0 volume changes per day

accumulated on the walls and baffles inside the fermentor (Fig. 8), and the mycelium in the effluent line after $4\frac{1}{2}$ days plugged the back-pressure regulating valve. The first problem of mycelium accumulating inside the fermentor was never satisfactorily resolved; it appears to be an inherent limitation of continuous operation. The second problem was circumvented, or at least postponed, by substituting a vertically mounted Grinnell 'straight-way' diaphragm valve for the conventional Saunders type in the pressure regulating unit.

With this change in equipment and reduction of the medium feed rate to 0.5 volume changes per day, a second continuous penicillin fermentation (Fig. 9) was maintained successfully for 13 days, with a yield level between 400 and 1,000 units per ml. Contamination occurred at 13 days, at which time the penicillin yield dropped and the run was terminated. Although the pH remained relatively steady during continuous operation, there

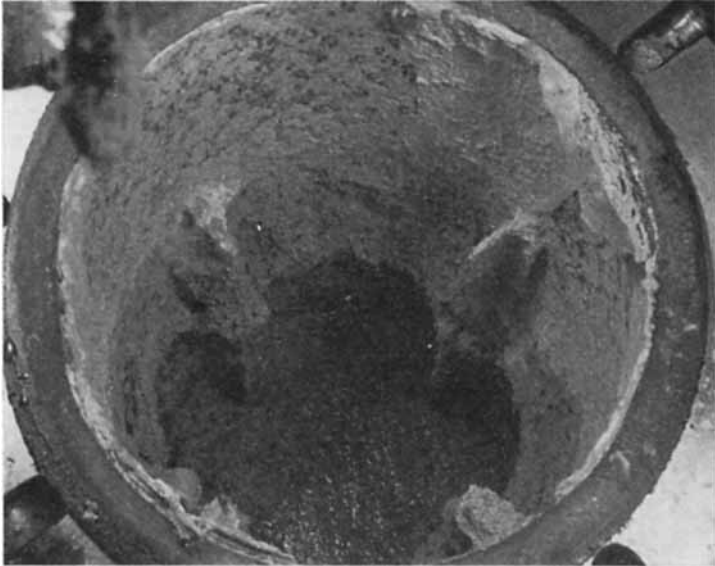


Fig. 8. View of accumulated mycelium in fermentor after 9 days of continuous penicillin fermentation

was considerable variation in the antibiotic level. As before, such fluctuations could usually be traced to an operational variable. For example, at points *A* and *B* on Fig. 9, the fermentor contents were observed to be foaming excessively; extra addition of antifoam agent was accompanied by an increased antibiotic level.

As shown in Fig. 10, a confirmatory run under the same conditions again reached and maintained for 3 days a penicillin level of 1,000 units per ml. However, another operational variable again apparently caused reduced yields twice during the 8-day

period of these operating conditions ; at points *A* and *B* on Fig. 10, a valve was observed to be operating defectively and allowing steam condensate to enter the fermentor. In both this and the preceding run, the level of total carbohydrates steadily and unexplainably diminished during continuous fermentation. After

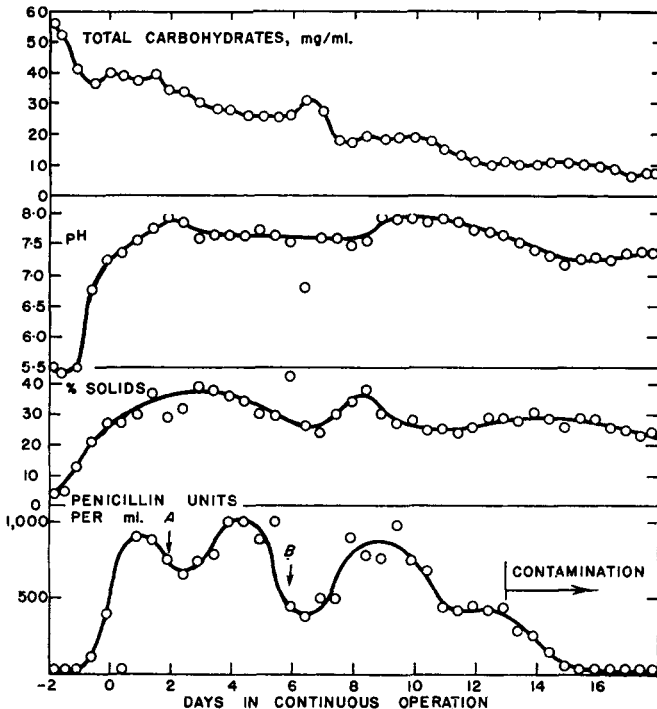


Fig. 9. Continuous penicillin fermentation at 0.5 volume changes per day

8 days, including recovery of the fermentation from the effects of the steam condensate, the medium being fed was changed so that 1.44 per cent glucose replaced the lactose originally employed. At the medium feed rate used, the glucose feed rate was 0.03 per cent per h. The use of glucose as a carbohydrate source at this feed rate was reported by Soltero and Johnson²⁹ to give better penicillin biosynthesis in batch fermentations. In our continuous operation, however, this change

was associated with a gradual reduction in penicillin, solids, and carbohydrates and with a rise in pH.

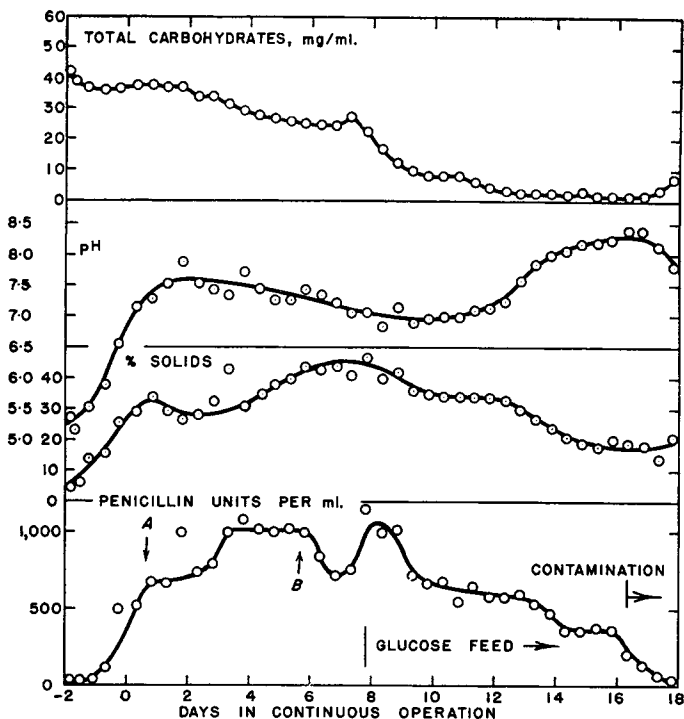


Fig. 10. Continuous penicillin fermentation at 0.5 volume changes per day, initially with lactose and then with glucose as the carbohydrate

Discussion and Conclusions

It appears that both chloramphenicol and penicillin can be produced by single-stage continuous fermentation processes. Using dilution rates of 1.0 and 0.5 volume changes per day, respectively, we were able to maintain both fermentations in the steady-state for periods in excess of 2 weeks with yields from $\frac{1}{4}$ to $\frac{1}{2}$ of the maxima we obtained in batch operation. In normal industrial practice, of course, production batches are harvested before the maximum is reached. Moreover, if the continuous yields are put on a daily production basis over a two-week operating period, they become roughly equal to or, in the case of

penicillin, almost twice those achieved over the same period by batch operation with its characteristic shutdowns. It seems likely that further development of the continuous process could make its daily yield considerably more favourable. However, the relative practicability of the two types of operations depends on a number of features other than yields, so that further comparisons on the basis of the limited data so far available are not worth while.

A prominent feature of both of the continuous fermentations was the fluctuation, particularly in antibiotic level, with the passage of time. Finn and Wilson⁸ have reported that the continuous growth of yeast may induce a sinusoidal cycling of the population, which will normally decay exponentially. In our continuous antibiotic fermentations, the oscillations usually could be traced to some operational deviation and did not appear to be inherent in the operation. The occurrence of plateaux during periods of several days in some of the experiments suggested that better controls should permit the attainment of true steady-state fermentations with antibiotic production levelling at the peaks of the observed oscillations.

The finding that continuous fermentations of penicillin required a lower dilution rate than those of chloramphenicol was expected, since moulds usually grow more slowly than actinomycetes. Totally unexpected was the observation that a lower continuous dilution rate lowered rather than raised the chloramphenicol yield. Since this occurred at the end of a run and since replacement of the original population with a mutant strain has been predicted to be inevitable in continuous culture,²² the lowered yield level might have been due to 'strain degeneration' rather than to the lowered feed rate. An experiment to test this idea, however, indicated otherwise. Moreover, the result could not be traced to contamination or to the accumulation of toxic factors, since a return to a batch operation resulted in a rise in the yield. Since it was not accompanied by reduced mycelial solids, the reduced yield apparently was the result of a factor limiting only the biosynthesis of the antibiotic. The phenomenon requires further study.

The accumulation of *Penicillium* mycelium on surfaces inside the fermentor and within pipes and valves may prove to be an

inherent and ultimate limitation of continuous production of penicillin. The situation in pipes and valves may be circumvented and postponed by appropriate modification of equipment. Conceivably, the mycelium retained within the fermentor may even contribute significantly to the steady-state yield level. Eventually, however, the resulting congestion will force cessation of the continuous operation, unless some method can be devised to prevent it.

Contamination with extraneous micro-organisms was common to all of the continuous fermentations conducted and represents another important limiting factor in successful continuous operation. Its control ultimately depends on refinements in equipment and handling, but certainly is possible. Although we operated aseptically for periods in excess of 2 weeks, other groups have conducted continuous bacterial cultures in a similar pilot plant for periods of several months.¹⁵ In almost every instance, we could trace contamination to the failure of some part of the equipment. Correction of such sources was and continues to be an important part of the development, e.g. replacing a fragile mechanical seal on the agitator shaft with a stuffing box. The larger, permanent equipment, accumulated experience, and possible integration of continuous sterilization with continuous fermentation should obviate many of the present problems of contamination if continuous fermentation becomes adopted in the fermentation industry.

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Mrs. Jo-Ann Cherry, Mr. D. M. Gallup, and Miss Sara S. DeLong conducted the analyses.

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